Attorney Docket No.: 6092.200-US PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Sjoholm et al. Confirmation No: 9041

Serial No.: 09/779,334 Group Art Unit: 1651

Filed: February 8, 2001 Examiner: Jon Weber

For: Use of Acid Stable Protease in Animal Feed

DECLARATION UNDER 37 CFR 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Peter Rahbek Østergaard, do hereby state and declare that

1. I obtained a Master of Science degree in Biochemistry from the University of Copenhagen in 1989. Since August 1, 1991, I have been employed by Novozymes A/S (formerly Novo Nordisk A/S). Currently, I am a Principal Scientist in the Protein Chemistry department. My responsibilities include purification and characterization of different enzymes including proteases.

2. The experiments described in this declaration were performed to determine if subtilisins derived from *Bacillus subtilis* and *Bacillus licheniformis* are acid stable, as defined in U.S. application no. 09/779,334.

3. The type strain of *Bacillus subtilis* (available from the American Type Culture Collection (United States), accession number ATCC 6633) and of *Bacillus licheniformis* (available from the National Collections of Industrial, Food and Marine Bacteria (United Kingdom), accession no. NCIB 9375), and a strain of *Bacillus subtilis var. natto* (obtained in 1972 from the Institute of Applied Microbiology (Tokyo, Japan), accession no. IAM 1033) were used for the production of protease preparations. A type strain is a reference strain for a species and generally used for comparison in the literature. The species *Bacillus natto* has been described in the literature. However, today *natto* is no longer considered a species of *Bacillus*, but a variant of *Bacillus subtilis*. Therefore, a type strain of *Bacillus natto* does not exist. A typical strain of

metalloproteases, subtilisins, and other serine proteases. However, the largest protease component in *Bacillus subtilis* and *Bacillus licheniformis* strains is a subtilisin.

4. The three *Bacillus* strains were grown in two different media: PS-1 and SKPX, which are known to be conducive to high protease production in *Bacillus* cells. See Calik et al., 2003, Enzyme and Microbial Technology, 33: 975-986 and Han-Seung et al., 2002, Process Biochemistry, 38: 155-159. The culture broths were centrifuged and filtered through a 0.45 micron filter to give clear supernatants. Two protease preparations from each *Bacillus* strain were obtained.

5. The pH-stability of the subtilisins contained in the six protease preparations was determined according to the procedure described in U.S. application no. 09/779,334 (in particular page 7, lines 10-29 and Example 2C), except that (x) a supernatant containing the subtilisins was used instead of a pure protease sample and (y) twenty microliters of the subtilisin-containing supernatant (diluted in 0.01% Triton X-100) was mixed with 100 microliters of the assay buffer; and the assay was started by adding 100 microliters Suc-AAPF-pNA substrate (50 mg dissolved in 1.0 ml DMSO and further diluted 45x with 0.01% Triton X-100). All known subtilisins are able to degrade Suc-AAPF-pNA, which is used in the assay. These differences would result in a greater acid-stability because the protease stability is improved in a supernatant than as pure protease, due to substrate stabilization and a lower protease concentration in the supernatant. Therefore, if a subtilisin contained in the supernatant is not acid-stable according to this procedure, then the pure subtilisin also would not be acid-stable according to the procedure described in U.S. application no. 09/779,334.

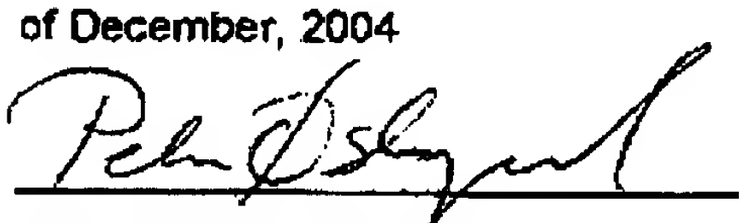
6. The results of the pH-stability studies of the subtilisins contained in the protease preparations are as follows:

B. licheniformis grown in PS-1:	0.05%
B. licheniformis grown in SKPX:	0.14%
B. subtilis var. natto grown in PS-1:	0.00%
B. subtilis var. natto grown in SKPX:	0.35%
B. subtilis grown in PS-1:	0.07%
B. subtilis grown in SKPX:	0.04%

contained in the protease preparations are acid-stable according to the definition of U.S. application no. 09/779,334.

7. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that the statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed this 15th day
of December, 2004



Peter Rahbek Østergaard